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Cyclin A2 Is Required for Sister Chromatid Segregation, But Not Separase Control, in Mouse Oocyte Meiosis

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SUMMARY

In meiosis, two specialized cell divisions allow the separation of paired chromosomes first, then of sister chromatids. Separase removes the cohesin complex holding sister chromatids together in a stepwise manner from chromosome arms in meiosis I, then from the centromere region in meiosis II. Using mouse oocytes, our study reveals that cyclin A2 promotes entry into meiosis, as well as an additional unexpected role; namely, its requirement for separase-dependent sister chromatid separation in meiosis II. Untimely cyclin A2-associated kinase activity in meiosis I leads to precocious sister separation, whereas inhibition of cyclin A2 in meiosis II prevents it. Accordingly, endogenous cyclin A is localized to kinetochores throughout meiosis II, but not in anaphase I. Additionally, we found that cyclin B1, but not cyclin A2, inhibits separase in meiosis I. These findings indicate that separase-dependent cohesin removal is differentially regulated by cyclin B1 and A2 in mammalian meiosis.

INTRODUCTION

Meiotic divisions are specialized cell divisions producing haploid gametes from a diploid precursor cell through two rounds of chromosome segregation without an intervening S-phase. In meiotic prophase, paired chromosomes are held together through chiasmata coming from recombination events between maternal and paternal chromosomes. Sister chromatids are linked through the cohesin complex in the arm and centromere region. In meiosis I, paired chromosomes are segregated to the opposite poles of the meiotic spindle, whereas in meiosis II, sister chromatids are segregated (Petronczki et al., 2003). For both divisions, cohesion has to be removed by separase at the metaphase-to-anaphase transition. Importantly, this is a two-step process: In meiosis I, only cohesin on arms is removed to allow the resolution of chiasmata and the separation of chromosome arms that have recombined. Centromeric cohesin, which is protected from cleavage in meiosis I, is then cleaved in meiosis II to allow sister chromatid separation (Petronczki et al., 2003). Protection of centromeric cohesin is mediated by Sgo2-dependent PP2A recruitment to centromeres. PP2A has been shown to dephosphorylate the cohesin subunit Rec8 and thereby prevent its cleavage by separase in *S. cerevisiae* (Clift and Marston, 2011; Kitajima et al., 2006; Riedel et al., 2006). How centromeric cohesin can be removed when cells progress through meiosis II is not entirely clear.

It is crucial that separase activity is tightly controlled until all chromosomes are correctly attached and aligned at the metaphase plate. In mouse oocyte meiosis I, separase is inhibited by securin until satisfaction of the Mad2-dependent spindle assembly checkpoint and activated at the metaphase-to-anaphase transition through anaphase-promoting complex or cyclosome (APC/C)-dependent degradation of securin (Herbert et al., 2003; McGuinness et al., 2009; Terret et al., 2003; Wassmann et al., 2003). Antibody injection experiments to block separase interaction with cyclin B1 indicated that separase activity is additionally inhibited by high cyclin B1-Cdk1 activity in oocyte meiosis I (Gorr et al., 2006). In addition to securin, the APC/C also targets cyclin B1 for degradation in meiosis I, and upon fertilization in meiosis II.

Whereas the importance of cyclin B1-associated kinase activity for oocyte meiosis has been well established, close to nothing is known about the possible roles of another M-phase cyclin, namely cyclin A. In higher vertebrates, two A-type cyclins exist: cyclin A1 and A2. Cyclin A2 has been shown to be expressed in the female germline. Its role is unknown because a complete knockout of cyclin A2 is lethal, and huge amounts of maternal stocks of mRNA encoding for cyclin A2 are present in oocytes and allow preimplantation development (Murphy et al., 1997; Persson et al., 2005; Winston et al., 2000; Wolgemuth, 2011). In somatic cells, cyclin A2 is required for correct

entry into mitosis, but essential for cell cycle progression only in hematopoietic and embryonic stem cells (Kalaszczynska et al., 2009).

In this study, we examined the role of cyclin A2 for the meiotic divisions in mouse oocytes. We show that cyclin A2 induces entry into meiosis I, and that interfering with cyclin A function in meiosis II inhibits sister chromatid separation. Our study reveals an unexpected role for cyclin A2 in mediating sister chromatid separation in meiosis II.

RESULTS AND DISCUSSION

Inhibition of Endogenous Cyclin A Prevents Sister Separation in Meiosis II

Endogenous cyclin A is present in germinal vesicle (GV) stage and metaphase II oocytes. A weak cyclin A signal can be detected by western blot analysis in GV oocytes and oocytes arrested in metaphase II, as previously reported (Persson et al., 2005; Winston et al., 2000). In meiosis I, most but not all (see below) endogenous cyclin A is degraded in prometaphase I (Figures 1A, 1B, S1A, and S1B).

To elucidate a potential role of cyclin A2 during meiosis I and II, we interfered directly with endogenous cyclin A through injection of two different anti-cyclin A antibodies into GV and metaphase II oocytes. GV oocytes were induced to enter meiosis I, and time of germinal vesicle breakdown (GVBD) as a marker for entry into the first meiotic division was determined. Chromosome spreads were performed in metaphase II to address whether chromosomes have correctly segregated in meiosis I. Anti-cyclin A antibody injection delayed entry into meiosis I, in contrast to control immunoglobulin G (IgG) or epitope-blocked antibody injections. Purified cyclin A2 protein injections rescued GVBD onset in antibody-injected oocytes (Figure 1C). No obvious effect on chromosome segregation in the first meiotic division was observed in oocytes injected with anti-cyclin A antibody (Figure 1D).

To analyze meiosis II, metaphase II oocytes were injected with the indicated antibodies, activated with strontium to induce the second meiotic division, and analyzed by chromosome spreads at a time when control oocytes had separated sister chromatids. In contrast to meiosis I, injection of the two cyclin A antibodies, but not control injections, prevented sister separation in meiosis II. Sister separation was rescued when recombinant cyclin A2 protein was injected (Figures 1E, and 1F, and S1C). The cyclin B1-GFP degradation profile after activation shows that the failure to segregate sisters was not due to a failure to exit metaphase II, because cyclin B1-GFP was degraded after activation (Figure 1G). Injection of antibodies against the related cyclin E1 did not affect sister chromatid separation in meiosis II (Figure S1D), demonstrating that the observed phenotype is specific for cyclin A. Furthermore, we could show that even though the mouse cyclin A antibody immunoprecipitates cyclin A from cell extracts (Figure S1E), it prevents in vitro phosphorylation of histone H1 (Figure S1F). This means that the mouse antibody blocks the assembly of active kinase complexes, or kinase activity. Therefore, we used the mouse antibody for subsequent experiments.

To follow chromosome movements during the second meiotic division in oocytes injected with cyclin A antibody by live imaging, oocytes were additionally injected with histone H2B-RFP in GV stage. Whereas control-injected oocytes separate sister chromatids and extrude a second polarbody (PB), oocytes injected with either mouse or rabbit (not shown) anti-cyclin A antibody cannot separate sister chromatids, or extrude a PB (Figure 1H). Our data indicate that cyclin A is required for sister chromatid segregation in meiosis II, but not for chromosome segregation in meiosis I.

Inhibition of Overall Cdk Activity in Meiosis II Prevents Sister Separation

Cdk1 has to be inactivated and its substrates dephosphorylated to exit metaphase II upon fertilization (Von Stetina and Orr-Weaver, 2011). It has been shown previously that inhibition of Cdk-associated kinase activity with the well-known Cdk inhibitor roscovitine (Meijer et al., 1997) activates metaphase II oocytes to exit meiosis II, but it has not been addressed whether sisters actually separate under these conditions (Phillips et al., 2002). Another study showed that roscovitine treatment alone does not induce chromatid separation in metaphase II oocytes, possibly because securin has not been degraded (Nabti et al., 2008). If cyclin A2-associated kinase activity were required for sister separation in meiosis II, then activation of oocytes under conditions where both cyclin A- and cyclin B-associated kinase activity are inhibited would lead to meiosis II exit with a failure to separate sisters. To address this point, we treated metaphase II-arrested oocytes with roscovitine and activated oocytes, which were then analyzed by chromosome spreads and live imaging. In the absence of roscovitine, oocytes correctly segregate their sister chromatids, whereas no sister separation took place in the presence of roscovitine (Figures 2A and 2B), even though cyclin B1-GFP was degraded, indicating exit from meiosis II (Figure 2C). Cdk activity is therefore required for sister chromatid separation in anaphase II.

Stable Cyclin A2 Expression Induces Precocious Sister Chromatid Separation

As is the case with other APC/C substrates, overexpression of wild-type cyclin A2 inhibits PB extrusion in meiosis I. Around 80% of oocytes that enter meiosis I remain arrested in metaphase I (Figures S2A and S2B). Exogenously expressed cyclin A2 is degraded in an APC/C-dependent manner in oocyte meiosis I (McGuinness et al., 2009). If cyclin A2 had a role in inducing sister chromatid separation in meiosis II, then its inappropriate presence due to expression of a nondegradable mutant throughout the metaphase-to-anaphase transition in meiosis I should lead to precocious sister separation. To this end, mRNAs coding for two stable versions of cyclin A2 were injected into mature, GV-arrested mouse oocytes: ΔNCyclin A2, a completely stabilized form of cyclin A2 harboring a 157-aa deletion of the N terminus (Fung et al., 2005), and ΔDBCyclin A2 with a deletion of the destruction box (den Elzen and Pines, 2001), but still not completely stabilized (Fung et al., 2005). For comparison, ΔDBCyclin B1 (also named Δ90Cyclin B1), which has a deletion of the destruction box and is completely stabilized (Holloway et al., 1993), was expressed. Stable cyclin B1 induces spontaneous entry into meiosis I only after extended incubation times (Reis et al., 2006). Both stable cyclin A2 mutants induce

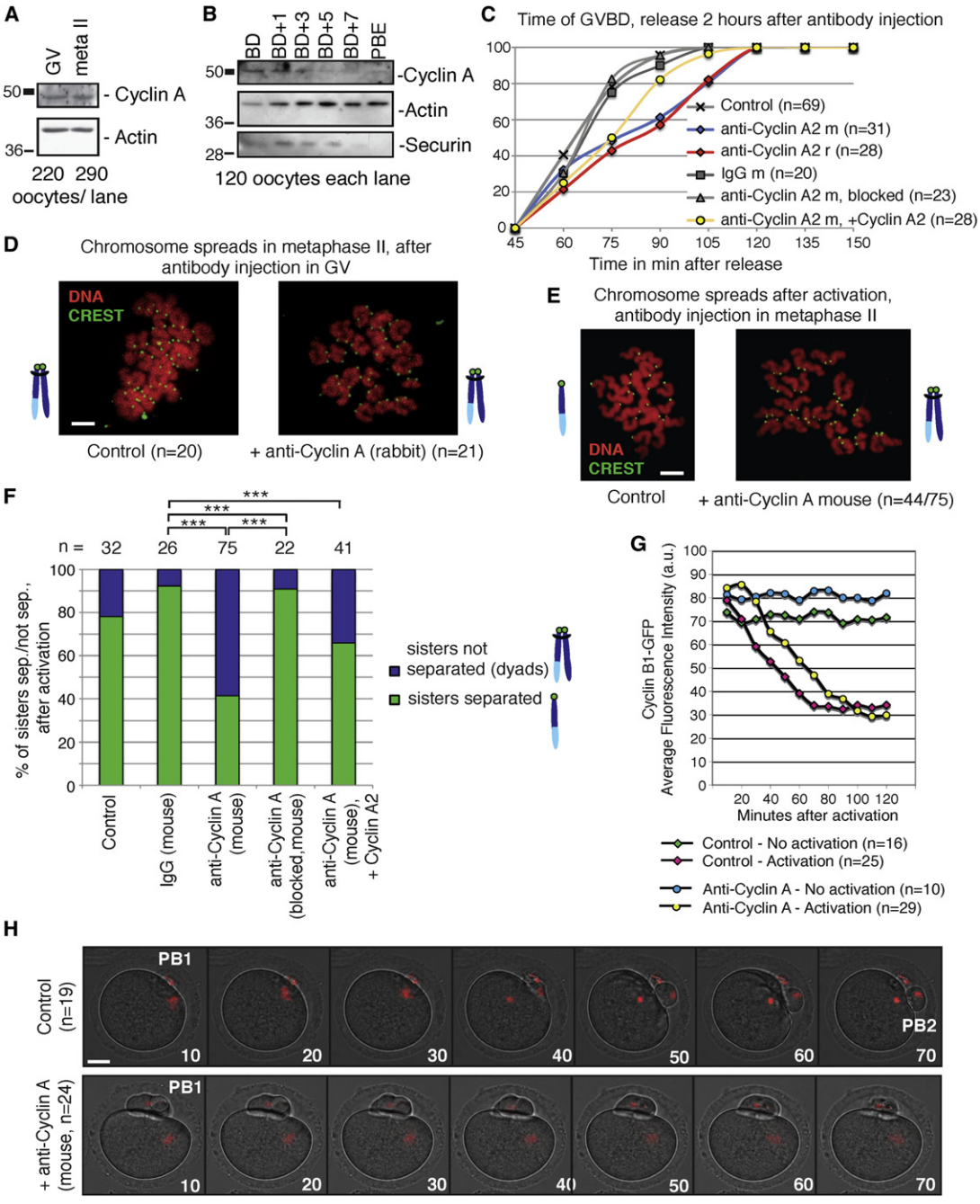


Figure 1. Inhibition of Endogenous Cyclin A Delays GVBD and Prevents Metaphase-to-Anaphase Transition in Meiosis II

(A) Endogenous cyclin A can be detected by western blot analysis in GV and metaphase II oocytes. The number of oocytes used per lane is indicated.

(B) Cyclin A western blot in meiosis I.

(C) Time of GVBD after release in oocytes injected with indicated antibodies (m, mouse; r, rabbit), or controls.

(D) Metaphase II chromosome spreads of oocytes injected in GV stage with anti-cyclin A antibodies as indicated. Kinetochores are stained with CREST (green), chromosomes with propidium iodide (red).

(E) Oocytes were injected with anti-cyclin A antibodies in metaphase II and activated. Chromosome spreads as in (D) after anaphase II, 50 min after activation.

(F) Percentage of sister separation observed in (E). The antibody injection results were assessed by variance analysis and found to be highly significant (***$p < 0.001$) when compared as indicated.

(G) Representative quantitations of cyclin B1-GFP fluorescence levels in meiosis II oocytes that were injected with anti-cyclin A antibodies in metaphase II, and activated as indicated.

(H) Selected time frames of representative movies of oocytes expressing histone H2B-RFP that were injected in metaphase II with control or anti-cyclin A antibodies. See also Figure S1.

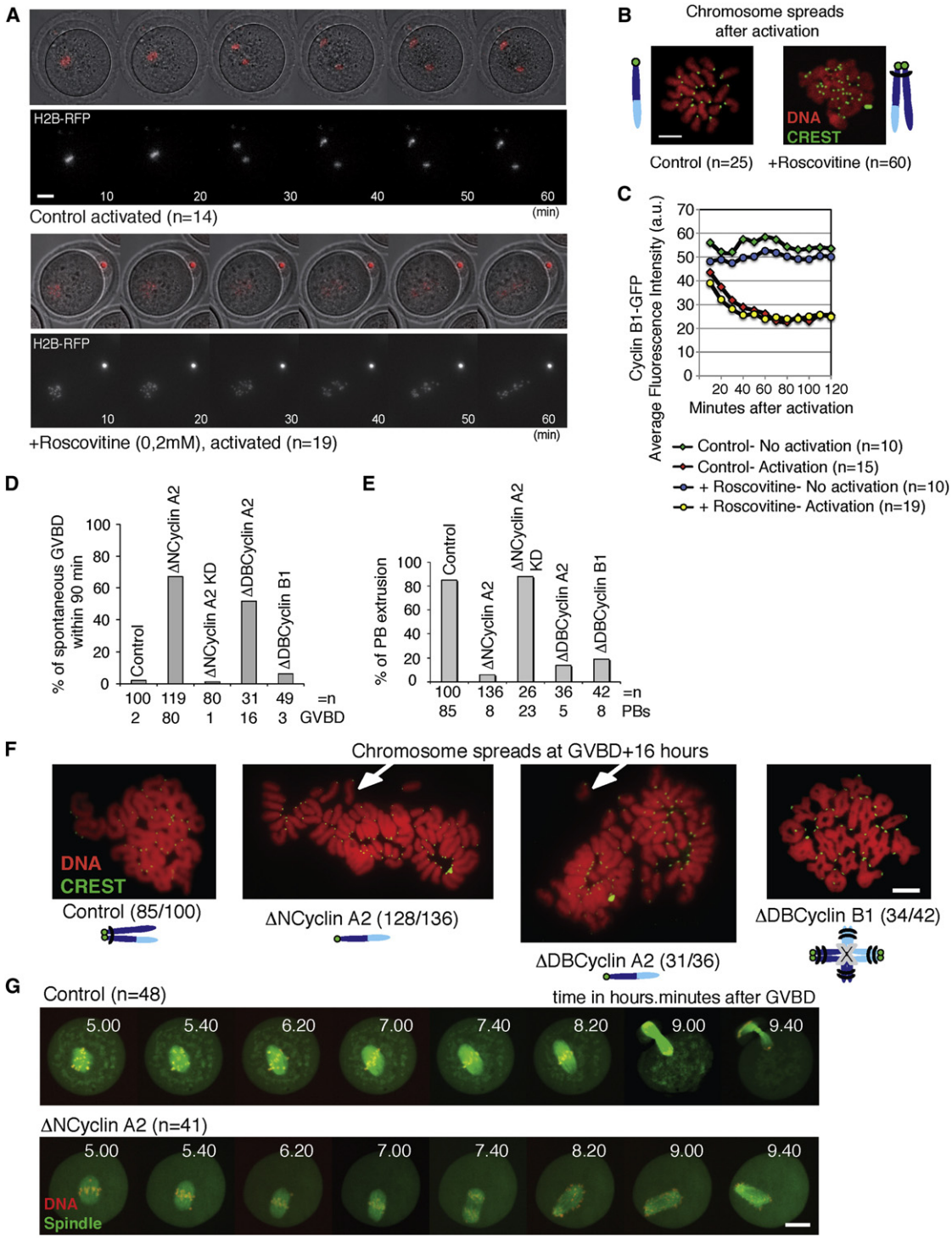


Figure 2. Inhibition of Overall Cdk Activity Prevents Sister Chromatid Separation in Meiosis II, whereas Expression of Stable Cyclin A2 Induces Sister Separation in Meiosis I

(A) Selected time frames of a representative movie of oocytes undergoing metaphase-to-anaphase transition of meiosis II in the presence of roscovitine where indicated. Chromosomes are visualized with H2B-RFP. Time after activation is indicated in minutes. Scale bar represents 20 μm.

(B) Representative chromosome spreads after activation, in the presence of roscovitine where indicated. Kinetochores are stained with CREST (green), and chromosomes with propidium iodide (red). CREST dots are still paired in the presence of roscovitine, indicating that sister chromatids have not been separated. Scale bar represents 5 μm.

(C) Representative quantitations of cyclin B1-GFP fluorescence levels in meiosis II oocytes treated with roscovitine and activated where indicated.

spontaneous GVBD within 90 min after injection (Figure 2D), confirming the findings of the antibody injection experiments where a delay in GVBD was observed. Therefore, we conclude that cyclin A2 promotes entry into meiosis I.

To analyze meiosis I chromosome segregation, oocytes were released to enter meiosis I in a synchronized manner immediately after injections. Stable cyclin A2 and B1 expression prevents PB extrusion during the first meiotic division (Figure 2E). However, chromosome spreads revealed that ΔNCyclinA2-injected and ΔDBCyclinA2-injected oocytes had separated sister chromatids, whereas ΔDBCyclin B1-expressing oocytes did not separate chromosomes or sister chromatids (Figure 2F), in agreement with a previous study where ΔDBCyclin B1-expressing oocytes were shown to remain arrested in metaphase I (Herbert et al., 2003). Our data indicate that constitutive presence of cyclin A2, but not cyclin B1, induces sister separation in meiosis I.

Constitutive Presence of Cyclin A2 Activity Prevents Exit from Meiosis I

To gain insight into why sister separation and no PB extrusion was observed upon expression of stable cyclin A2, oocytes co-expressing histone H2B-RFP and β-tubulin-GFP were followed by time-lapse microscopy throughout the first meiotic division. Expression of stable cyclin A2 allows oocytes to undergo metaphase-to-anaphase transition of meiosis I (Figure 2G; Movies S1 and S2), similar to what has been described in mitotic cells for stable cyclin A2 and low levels of stable cyclin B1 expression (Geley et al., 2001). Importantly, oocytes are then blocked in an anaphase I-like state without being able to complete separation of chromosomes to the opposite poles of the spindle, or to extrude a PB. Instead, chromosomes oscillate along the spindle, and move back and forth between poles (Figures 2G and S2C; Movies S1 and S2). It is important to note that prometaphase I progression occurs normally, and a metaphase plate is established (Figures 2G, S2C, and S2D; Movies S1 and S2). As expected, stable cyclin B1-expressing oocytes do not progress beyond metaphase I (Movie S3).

To confirm that stable cyclin A2-injected oocytes do not continue into meiosis II, we checked whether securin and cyclin B1 reaccumulate in meiosis II after their degradation at the metaphase-to-anaphase transition in meiosis I (Herbert et al., 2003; McGuinness et al., 2009). In stable cyclin A2-injected oocytes, the degradation of exogenously expressed YFP-securin and cyclin B1-GFP takes place, but not their reaccumulation, in contrast to control oocytes (Figures S2E and S2F). This indicates that oocytes do not progress into meiosis II and instead remain in an anaphase I-like state.

Does the phenotype observed upon stable cyclin A2 expression depend on cyclin A2-associated kinase activity? To answer this question, stable cyclin A2 with essential sites for interaction with its Cdk subunit mutated (Zhu et al., 2004) was expressed. This "kinase-dead," stable cyclin A2 (ΔNCyclin A2 KD) did not affect entry into, progression through, or exit from meiosis I (Figures 2D, 2E, and S2G). Thus, the ability of stable cyclin A2 to prevent cell cycle progression beyond anaphase I is due to associated Cdk activity and not the cyclin per se. Consequently, artificially inhibiting Cdk activity should allow these oocytes to exit anaphase I. To address this point, cyclin A2-associated kinase activity was inhibited with roscovitine. Oocytes expressing stable cyclin A2 were allowed to progress into anaphase I, where they remained with oscillating chromosomes, before roscovitine was added. Real-time imaging revealed that these oocytes complete anaphase I and remain blocked in telophase (Figure S2H). We did not observe PB extrusion, probably because oocytes were held for too long in anaphase I, and no stable spindle could be maintained due to precocious separation of sister chromatids.

Precocious Sister Chromatid Separation Takes Place in Anaphase I

The different phenotypes obtained upon expression of wild-type or stable cyclin A2 could have been due to important differences in expression levels. Figure S3A shows that this is not the case and that both mutants are expressed at comparable levels in prometaphase I. The different phenotypes upon stable cyclin A and B expression were also not due to different expression levels, because both stable GFP-tagged cyclin A2 and cyclin B1 are expressed at similar levels (Figure S3B). Additionally, injection of ten times lower amounts of stable cyclin B1 mRNAs did not result in an anaphase I-like phenotype (Figure S3C), unlike in mitosis (Geley et al., 2001).

The observed sister chromatid separation in stable cyclin A2-expressing oocytes was either due to premature loss of centromeric cohesin in meiosis I, or uncoupling of cohesin deprotection from meiosis I exit after extended periods of time. Thus we examined if the proportion of sister chromatids that were separated was different when chromosome spreads were obtained immediately after anaphase I onset, or at a later time point, when control oocytes were in metaphase II. Chromosome spreads and high-resolution movies around the time of anaphase onset showed that sisters are separated at the same time as bivalents (Figures 3A [the spread at GVBD + 8 hr shows an anaphase I with sisters already separated], 3B, and 3C; Movies S4 and S5). On the other hand, the proportion of sisters separated was dependent on the expression levels of stable cyclin A2 (Figure 3D). These results indicate that the presence of cyclin A2-Cdk activity at the metaphase-to-anaphase transition in meiosis I leads to loss of centromeric cohesin, which

(D) Percentage of oocytes injected with the indicated mRNAs that underwent spontaneous GVBD within 90 min after injection in dbcAmp containing medium.
(E) Percentage of oocytes injected with the indicated mRNAs that extruded PBs.
(F) Chromosome spreads 16 hr after GVBD of oocytes expressing the indicated cyclin mutants. Kinetochores are stained with CREST (green), and chromosomes with propidium iodide (red). The arrows mark examples of single sister chromatids. Scale bar represents 5 μm.
(G) Live imaging of oocytes that have been coinjected with histone H2B-RFP to visualize chromosomes, β-tubulin-GFP to visualize spindles, and ΔNCyclin A2 where indicated. Selected time frames (every 40 min) of collapsed z-sections (ten sections, 3 μm steps), from a representative spinning disk confocal movie of a control and a ΔNCyclin A2-expressing oocyte are shown. Time points after GVBD are indicated. Scale bar represents 20 μm.
See also Figure S2.

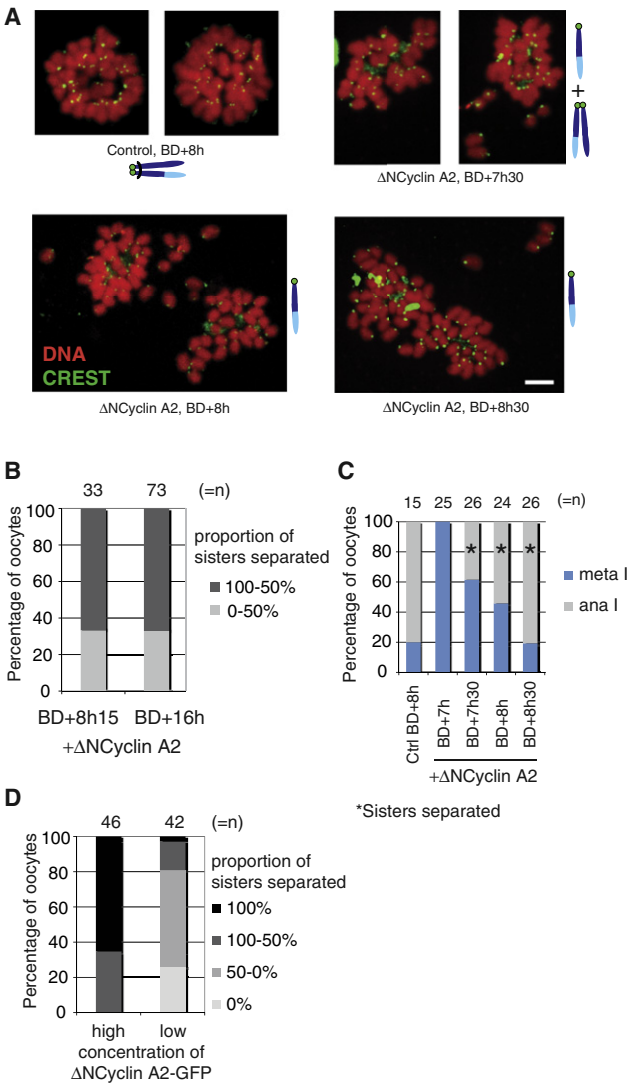


Figure 3. Stable Cyclin A2 Induced Precocious Sister Chromatid Separation in Meiosis I

(A) Chromosome spreads of oocytes injected with ΔNCyclin A2 mRNAs where indicated. Spreads were performed at the indicated time points after GVBD, at metaphase-to-anaphase transition. At GVBD + 8 hr, an example of an anaphase I is shown. Kinetochores are stained with CREST (green), and chromosomes with propidium iodide (red). Scale bar: 5 μm. For number of oocytes analyzed see (C).

(B) Percentage of ΔNCyclin A2 expressing oocytes that had undergone the metaphase-to-anaphase transition of meiosis I harboring the indicated proportion of separated sister chromatids at 8, 15, or 16 hr after GVBD. Control oocytes extruded PBs with normal timing at 8–9 hr after GVBD (not shown).

(C) Percentage of oocytes at the indicated time points after GVBD that were in metaphase or anaphase, as determined by chromosome spreads. Important note: all ΔNCyclin A2-expressing oocytes contained at least 20% clearly visible separated single sister chromatids.

(D) Percentage of oocytes expressing low or high levels of ΔNCyclin A2-GFP with the indicated proportion of separated sister chromatids, as determined by chromosome spreads at GVBD + 16 hr.

See also Figure S3 and Movies S1, and S2, and S3.

should hold sisters together until metaphase II (Kitajima et al., 2006; Riedel et al., 2006).

Premature Sister Chromatid Separation in the Presence of Stable Cyclin A2 Depends on Separase

To gain insight into how cyclin A2 induces sister chromatid segregation, we asked whether the phenotype observed in the presence of stable cyclin A2 in meiosis I depends on separase. A truncated version of securin, that is not recognized by the APC/C but retains its inhibitory activity on separase (ΔDBSecurin) (Herbert et al., 2003), was expressed together with stable cyclin A2. Inhibition of separase with ΔDBSecurin converts the anaphase I-like state of stable cyclin A2-expressing oocytes into a metaphase I-like arrest (Figure 4A), similar to what is observed with stable cyclin B1 alone (Figure 2F). This indicates that sister separation depends on separase and, furthermore, that cyclin A2 cannot inhibit separase activity in meiosis I.

Next, we asked whether the metaphase I arrest upon stable cyclin B1 expression was due to inhibition of separase through cyclin B1, which was shown to control separase through phosphorylation in mitosis (Gorr et al., 2005). A nonphosphorylatable mutant of separase (PM-separase) was expressed to determine whether it rescues the metaphase I arrest of stable cyclin B1. Indeed, coinjection of PM-separase (Gorr et al., 2005), but not wild-type separase, with stable cyclin B1 allows for the separation of homologous chromosomes to occur (Figure 4B), suggesting that separase is inhibited by cyclin B1-Cdk1-dependent phosphorylation. As expected, no PBs are extruded, because high cyclin B1-associated Cdk activity prevents exit from meiosis I, and because nonphosphorylatable separase is not able to downregulate stable cyclin B1 kinase activity and function as a Cdk inhibitor (Gorr et al., 2006). Stable cyclin B1 and PM-separase double-injected oocytes remain in an anaphase I-like state with separated homologous chromosomes (Figure S4A), such as stable cyclin A2-expressing oocytes. Crucially though, we never observed precocious sister chromatid separation in these oocytes.

This led us to the question of whether the two cyclins have different capacities to interact with separase. To address this issue, stable cyclin A2 and/or B1 were expressed together with wild-type or PM-separase in human tissue culture cells. Copurification with separase shows that, indeed, only cyclin B1 and not cyclin A2 interacts with and therefore inhibits wild-type separase (Figure S4B). Consistent with this, oocytes from separase$^{F/F}$ Cre^{+} mice, which are devoid of separase (Kudo et al., 2006), cannot separate chromosomes in meiosis I in the presence of stable cyclin A2, just like stable cyclin B1-expressing oocytes (Figure 4C). We conclude that sister chromatid separation induced by stable cyclin A2 expression depends on separase.

Does stable cyclin A2 induce sister chromatid separation when injected into metaphase II oocytes? To address this question, metaphase II oocytes were injected with stable cyclin A2 mRNA and analyzed by chromosome spreads 2 hr later. Figure S4C shows that stable cyclin A2 expression indeed induces sister separation in meiosis II. To address whether separase is required for sister separation in meiosis II, oocytes from separase$^{F/F}$ Cre^{+} mice were induced to undergo meiotic maturation. Without separase, oocytes progress into metaphase II even

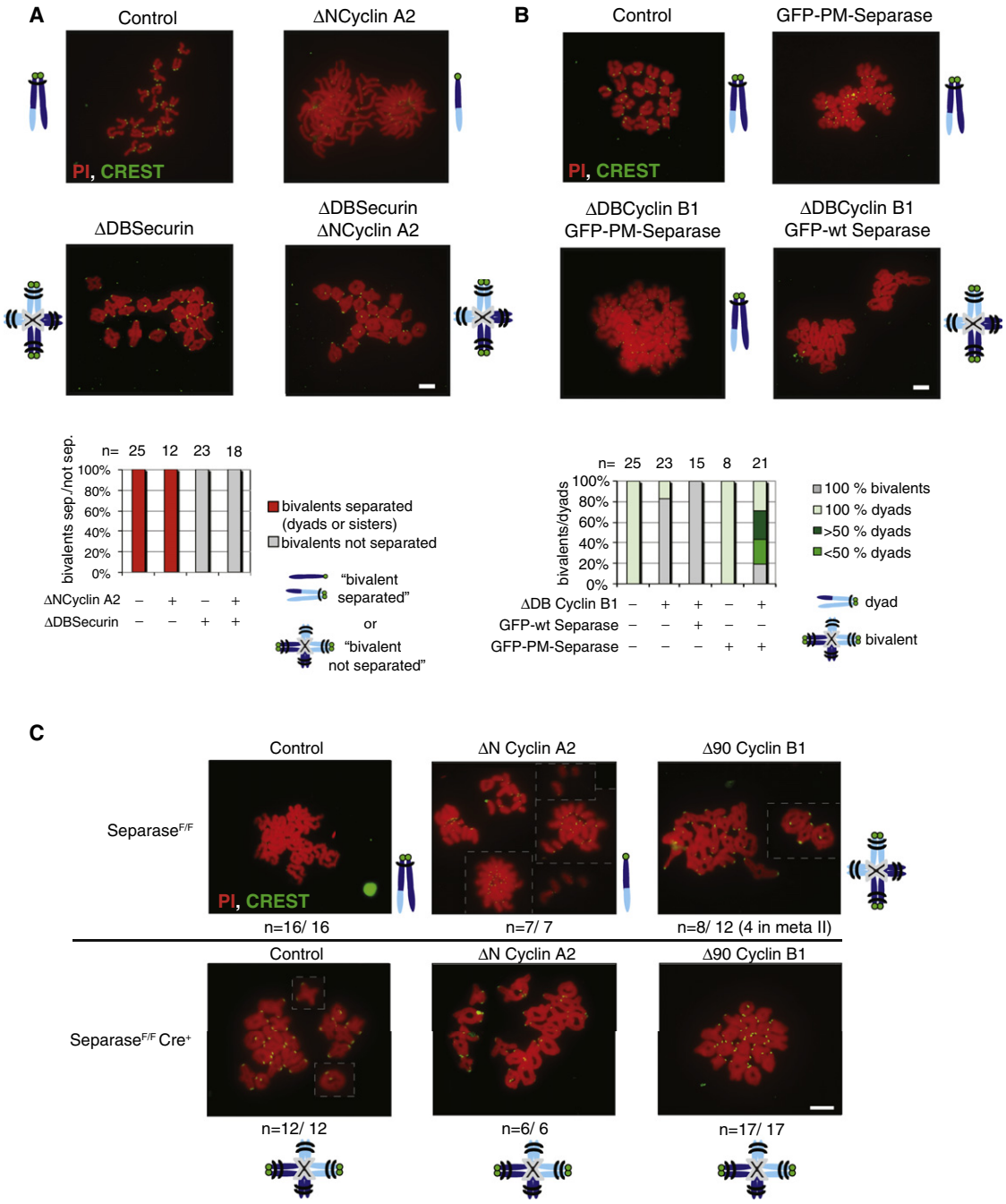


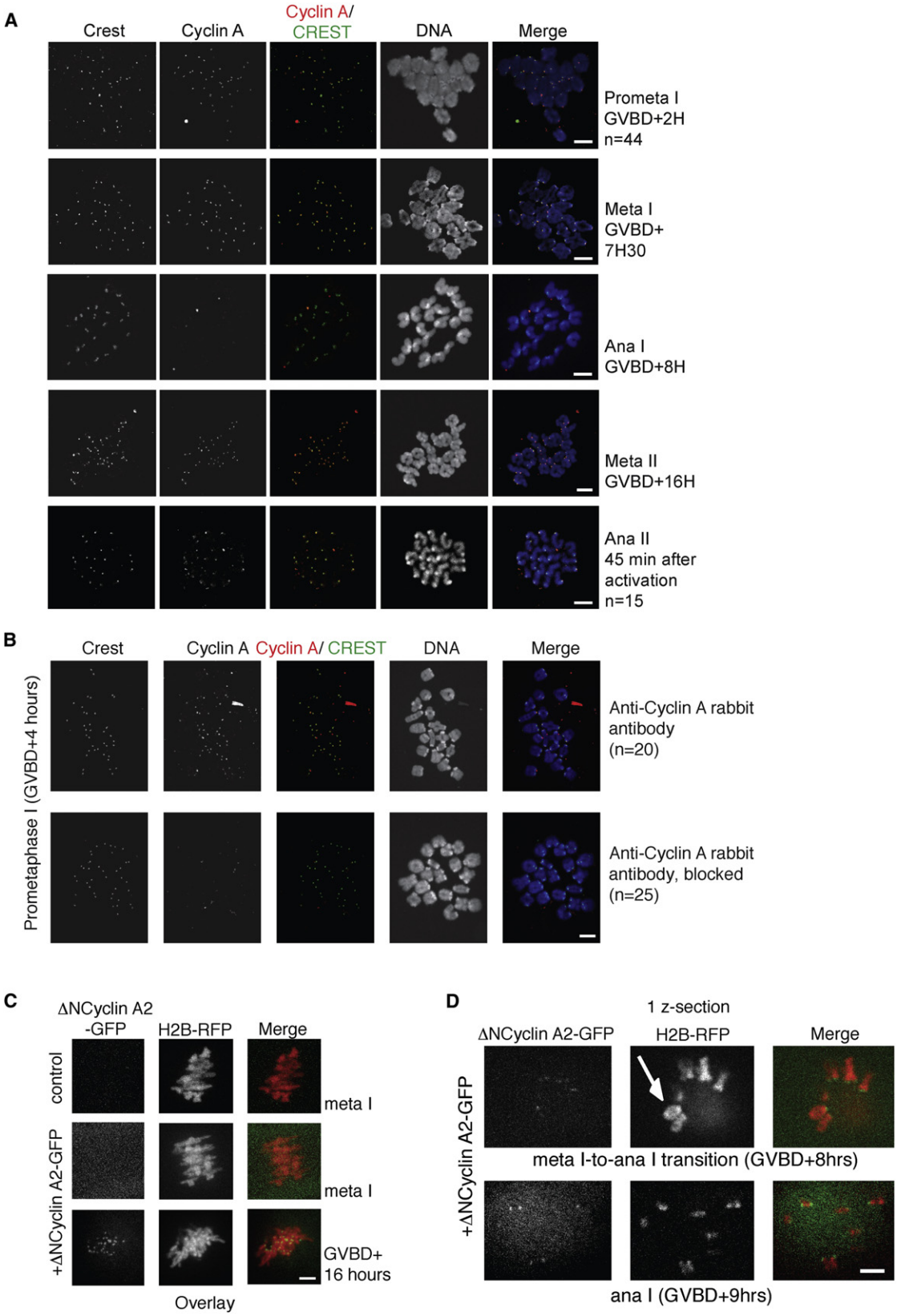
Figure 4. Only Stable Cyclin A2 Induces Sister Separation in a Separase-Dependent Manner in Meiosis I

(A) Chromosome spreads 16 hr after GVBD of oocytes expressing ΔNCyclin A2 and ΔDBSecurin as indicated. Kinetochores are stained with CREST (green), and chromosomes with propidium iodide (red). The graph below shows the percentage of oocytes with separated bivalents (either individual chromosomes, or sister chromatids), or bivalents that are not separated.

(B) Chromosome spreads as in (A) 16 hr after GVBD of oocytes expressing ΔDBCyclin B1, and GFP-wt separase or GFP-PM-separase as indicated. The graph below shows the percentage of oocytes with bivalents, or dyads. See also Figure S1 and Movies S1 and S3.

(C) Chromosome spreads of oocytes with the indicated genotype injected with the designated mRNAs, 14–16 hr after GVBD. The number of oocytes analyzed is indicated. Dashed lines show where images of one chromosome spread were assembled to minimize picture size.

Scale bars represent 5 μm. See also Figure S4 and Movie S4.


A
Crest
Cyclin A
Cyclin A/ CREST
DNA
Merge
Prometa I GVBD+2H n=44
Meta I GVBD+ 7H30
Ana I GVBD+8H
Meta II GVBD+16H
Ana II 45 min after activation n=15
B
Crest
Cyclin A
Cyclin A/ CREST
DNA
Merge
Prometaphase I (GVBD+4 hours)
Anti-Cyclin A rabbit antibody (n=20)
Anti-Cyclin A rabbit antibody, blocked (n=25)
C
ΔNCyclin A2 -GFP
H2B-RFP
Merge
control
+ΔNCyclin A2-GFP
meta I
meta I
GVBD+ 16 hours
Overlay
D
1 z-section
ΔNCyclin A2-GFP
H2B-RFP
Merge
+ΔNCyclin A2-GFP
meta I-to-ana I transition (GVBD+8hrs)
ana I (GVBD+9hrs)

though they are not able to segregate chromosomes in meiosis I (Kudo et al., 2006). Neither bivalents nor sister chromatids were separated in metaphase II separase$^{F/F}$ Cre^{+} oocytes injected with stable cyclin A2, showing that sister separation occurs in a separase-dependent manner.

Cyclin A2 Does Not Promote Bipolar Instead of Monopolar Attachment in Meiosis I

Precocious separation of sister chromatids in meiosis I could have been explained through a mechanism by which cyclin A2 would promote bipolar instead of monopolar kinetochore orientation. Chromosome spreads in meiosis I show that sister kinetochores appear as one dot and are not separated in the presence of stable cyclin A2, indicating monopolar attachment (Figure S5A). Cold stable microtubules are attached correctly in metaphase I oocytes expressing stable cyclin A2 (Figure S5B); therefore, we conclude that cyclin A2 does not inhibit monopolar attachment and thereby induce sister separation in meiosis I.

Cyclin A2 May Induce Sister Chromatid Separation in Meiosis II through a PP2A-Dependent Mechanism

How then does cyclin A2 induce loss of centromeric cohesin and sister separation in meiosis? Sgo2 localizes PP2A to the centromere region to prevent removal of centromeric cohesin in meiosis I. It was attractive to speculate that cyclin A2 affects localization of Sgo2/PP2A in meiosis II. We show here that this is not the case: expression of stable cyclin A2 in meiosis I (oocytes were maintained arrested in metaphase I with the proteasome inhibitor MG132) did not affect localization of Sgo2 or PP2A (Figure S5C). Sgo2 and PP2A were detected even on separated, single sister chromatids in the anaphase I-like state of stable cyclin A2-expressing oocytes without MG132.

On the other hand, cyclin A2 may regulate Sgo2/PP2A through a mechanism other than affecting their localization (through phosphorylation of PP2A or a PP2A regulatory protein, for example). In this case, inhibition of endogenous cyclin A was expected not to affect spontaneous sister chromatid separation observed upon inhibition of PP2A by okadaic acid in meiosis II. Indeed, okadaic acid treatment still induces sister chromatid separation in oocytes injected with anti-cyclin A antibody, suggesting that cyclin A2 may induce sister separation through a mechanism that requires PP2A (Figure S5D).

Endogenous Cyclin A Is Localized at Centromeres in Meiosis

If cyclin A has a role in inducing removal of centromeric cohesin, then we expected it to be localized to centromeres in meiosis II. We performed immunostaining on chromosome spreads to localize cyclin A more precisely. In meiosis II, endogenous cyclin A was indeed localized to centromeres in metaphase and anaphase. We also found cyclin A at centromeres in meiosis I from prometaphase to metaphase (Figures 5A and 5B), meaning that some cyclin A (Figure 1B) that has apparently escaped APC/C-dependent degradation is still found at centromeres. Importantly, cyclin A was not localized to centromeres in anaphase I (Figures 5A, 5B, S5E, and S5F). GFP-tagged stable cyclin A2 was not detected at kinetochores until metaphase I, probably because it is unable to compete with endogenous cyclin A. Accordingly, GFP-tagged stable cyclin A2 was detected by live imaging at centromeres at anaphase onset and in anaphase I (Figures 5C and 5D), when endogenous cyclin A has disappeared. Persistence of stable cyclin A2 at centromeres during anaphase I, together with loss of centromeric cohesin, is consistent with the hypothesis that cyclin A2-Cdk is active on centromeres at the metaphase-to-anaphase transition in meiosis II and phosphorylates an unknown substrate involved in centromeric cohesion removal, in order to promote chromatid separation in meiosis II.

In conclusion, our work shows the requirement of cyclin A2 for entry into meiosis I and for sister chromatid segregation in meiosis II in mouse oocytes. Future work will aim at identifying cyclin A2-Cdk-specific targets at the centromere that are phosphorylated in meiosis II to elucidate the molecular mechanisms of sister chromatid segregation in mammalian oocytes. Errors in chromosome segregation cause the generation of oocytes harboring the wrong number of chromosomes that can give rise to aneuploid embryos. In humans, defects in meiotic cell divisions have dire consequences, because aneuploid embryos either fail to develop to term or lead to the birth of trisomic individuals with sometimes severe developmental defects (Hassold and Hunt, 2001). Therefore, we need to understand the molecular mechanisms ensuring the generation of gametes with the correct ploidy.

EXPERIMENTAL PROCEDURES

In Vitro Oocyte Culture

Germinal vesicle stage oocytes were obtained from adult Swiss mice 10 to 16 weeks old (Janvier, France), or separase$^{f/f}$ Zp3Cre^{+} mice and Cre negative litter mates (Kudo et al., 2006). Oocytes were cultured in self-made M2 medium as described in (Hached et al., 2011). Roscovitine was used at 0.2 mM, okadaic acid at 600 nM, and MG132 was added 6 hr after GVBD at 5 μM, where indicated. Oocytes for Figures S2A and S3B were harvested as described in (Herbert et al., 2003). For parthenogenetic activation, oocytes were incubated 16 hr after GVBD in $CaCl_2$-free M2 medium containing 10 mM $SrCl_2$ (Sigma). Extrusion of a second PB was observed after 45 min. For chromosome spreads of activated oocytes, the zona pellucida was removed 14–15 hr after GVBD and before activation with Tyrode's acidic solution, and oocytes were left to recover.

Figure 5. Cyclin A Is Localized to Centromeres at All Stages of Meiosis Except Anaphase I

(A) Chromosome spreads were performed at the indicated times after GVBD and stained with CREST (green) and anti-cyclin A (red) antibodies. Chromosomes were stained with Hoechst (blue). Note: the CREST signal colocalizes with cyclin A2 at all stages except anaphase I.

(B) Spreads as in (A) at GVBD + 4 hr. Antibody control with epitope-blocked cyclin A antibody.

(C) Localization of ΔNCyclin A2-GFP at centromeres in anaphase I, shown by live imaging, at the indicated time points after GVBD. Chromosomes are visualized with H2B-RFP. Overlays of 15 z-sections (0.7 μm steps) of the individual channels and a merge are shown. (D) One individual z-section of each channel and their merge as in (C). The arrow indicates a bivalent chromosome with cyclin A staining just before anaphase I onset. A total of 16 control oocytes and 24 ΔNCyclin A2-GFP-expressing oocytes were repeatedly analyzed from GVBD + 7.5 hr to GVBD + 9 hr and again 16 hr after GVBD.

Scale bars represent 5 μm. See also Figure S5.

Antibody Microinjections

For meiosis I injections, indicated antibodies (0.5 mg/ml, diluted in PBS, injection of 1–10 pl) were injected into GV stage oocytes. Oocytes were incubated for 2 hr and then released into dbcAmp-free M2 medium. In meiosis II, oocytes were microinjected as above using a 40× objective with the indicated antibodies and incubated for 2–3 hr before activation. Control antibodies used were anti-Flag antibodies (mouse monoclonal [Sigma Aldrich F3040] and rabbit polyclonal [Santa Cruz sc807]), nonspecific purified IgGs (from mouse [Santa Cruz sc2025] and rabbit serum [Sigma Aldrich]), and cyclin E1 antibody (Cell Signaling 4132). Endogenous cyclin A was blocked with one of two different antibodies: a mouse monoclonal antibody (ab38, from Abcam) that recognizes a C-terminal cyclin A2 fragment containing the cyclin box starting with amino acid 172 (S. Geley, personal communication), and a rabbit polyclonal antibody (ab2097, from Abcam) that recognizes full-length cyclin A2. For controls, both cyclin A antibodies were epitope-blocked with purified recombinant GST-cyclin A protein (Abnova) bound to GST beads, and used for injections. Additionally, both cyclin A antibodies were digested with trypsin immobilized on Sepharose beads (Sigma), according to the manufacturer's suggestions, and used for injections. To rescue timing of meiosis I entry or sister chromatid segregation in oocytes injected with cyclin A antibody, purified recombinant cyclin A at 0.15 mg/ml was injected just prior to antibody injections.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.002.

LICENSING INFORMATION

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